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STUDIES ON CYTOCHROME C

AV
SVEN *PALÉUS*

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AKADEMISK AVHANDLING

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Studies on Cytochrome c

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I. A Comparative Study of Cytochromes c from Different Sources

Between 1882 and 1886, the Irish physician *MacMunn*^{1, 2} called attention to a pigment which he found in spectroscopic investigations of muscles and other tissues from different species throughout the animal kingdom, and which he named myo- or histohaematin. *MacMunn* believed that the pigment, which in the visible range showed four absorption bands in the reduced state, took part in the tissue respiration. Furthermore, he suggested that it ought not to be confused with the natural blood pigment haemoglobin, even if it was related to it. This work of *MacMunn* was very much criticized by some of his contemporaries, and his observations were forgotten until 1925, when *Keilin*³ confirmed his spectroscopic observations and showed that the pigment was widely distributed in plants, animals and aerobic bacteria. *Keilin* also changed the name of *MacMunn*'s pigment to "cytochrome". He attributed three of the *MacMunn* bands to three different compounds, cytochromes a, b and c. The fourth *MacMunn* band was common to all three cytochromes. At about this time the haemochromogen* nature of cytochrome was confirmed by *Anson* and *Mirsky*⁴.

Since their rediscovery by *Keilin*, the different cytochromes have been studied intensively and their important role in the transferring of electrons from metabolites to molecular oxygen verified, to a large extent by the fundamental discoveries of *Warburg*⁷. In the intracellular respiration he demonstrated the importance of the electron shift between reduced and oxidized iron present in an enzyme which he named "das sauerstoffübertragende Atmungsferment". Because of its firm attachment to the cell, *Warburg* was not able to isolate "the respiratory enzyme", but by studying the photodissociation of its CO-compound he was able to observe both its relative and absolute absorption spectrum. It later turned out that *Warburg*'s respiratory enzyme functions as a cytochrome oxidase. *Keilin*⁸ preferred to call it "cytochrome a₃" but the name cytochrome oxidase is now fairly generally accepted. The present concept of the physiological function of the cytochromes may be illustrated by the simplified version of the sequence⁹ of cellular oxidation of metabolites*:

* A haemochromogen (a name introduced by *Hoppe-Seyler*⁵) consists, according to *Anson* and *Mirsky*⁴, of haem (= ferroporphyrin) attached to nitrogenous bases. The ferrous iron is bound by covalent linkages, and the bonds to the pyrrole nitrogens lie in one plane, while the bonds to the base are perpendicular to this plane⁶.

metabolite $\rightarrow$ hydrogen transferring enzymes $\rightarrow$ cytochromes $\rightarrow$ cytochrome oxidase $\rightarrow$ O₂

For a more detailed discussion the reader is referred to recent reviews^{10, 11}.

Cytochrome c is the most studied of the different cytochromes due to its easy solubility and great stability over a wide pH-range. Although *MacMunn*² was able to extract "myohematin" in a modified form from some tissues, *Keilin* and coworkers^{12, 13} were the first to describe a partially purified cytochrome c preparation, in this case from baker's yeast. In 1936 *Theorell*¹⁴ worked out a method for large scale preparations of cytochrome c and at the same time gave an account of some of its physico-chemical properties. *Theorell*^{15, 16} also demonstrated that the haem in cytochrome c was linked to its protein part via two thioether bridges. In 1939 *Theorell* and *Åkeson*¹⁷ described an electrophoretic method for preparing pure cytochrome c, and later¹⁸ the same authors gave an extensive account of the chemical composition and physico-chemical properties of this preparation. *Paul*^{19, 20} was able to split cytochrome c with silver salts into a colourless protein and an optically active isomer of haematoximin. At about the same time, some peptic degradation products were obtained by controlled digestion of cytochrome c with different proteolytic enzymes²¹. The amino acid sequence of one of these peptides, the tryptic one, has recently been published by *Tuppy* and *Bodo*²².

When the present investigations were started some years ago, the conceptual picture of cytochrome c as postulated by *Theorell* and co-workers was as follows. The prosthetic group, a hematin disc, is imagined as being embedded in a crevice of the protein moiety, to which it is linked via thioether bonds. In addition to the four porphyrin nitrogen atoms, the iron in the prosthetic group is bound by covalent bonds to two nitrogenous haemochrome-forming groups, probably the imidazoles of histidine. The described structure of cytochrome c explains its non-oxidizability and inability to form CO- and cyanide compounds at physiological pH-values.

The original object of the present study was to find out possible differences between cytochrome c from different animal species, similar to those which had already been demonstrated in the case of haemoglobin^{23, 24}. During this investigation, a new method²⁵, convenient for the preparation of both small and large quantities of pure cytochrome c was developed. For this purpose one of the modern ion exchangers proved quite convenient and the first protein to be successfully purified with the aid of this method was cytochrome c. Preparations from beef, chicken and salmon hearts thus obtained were then compared with respect to their physico-chemical characteristics and chemical composition. Special attention was given to the correlation of the histidine content with the acid-base titration curves²⁶. Furthermore, a paper electrophoretic method²⁷

* The component at the right-hand side of each arrow is the oxidant for the reduced form of the compound at the left-hand side.

for the purification of cytochrome has been introduced, with the help of which some special properties of cytochrome c have been investigated. Because of the uncertainty about the exact number of cysteine residues²⁸ present in cytochrome c, the sulphur content of one of the preparations was determined²⁹. The sedimentation constants³⁰ of cytochromes prepared from different species and by different methods have been studied. A peptic product of cytochrome c from different animals, consisting of 11 amino acids, linked by two of its cysteine residues to hematin, has been obtained and the amino acid sequence determined³¹. Finally, some of the physico-chemical properties of this degradation product have been described and compared with those of cytochrome c itself³².

Purification

As previously mentioned the presence of cytochrome c was first demonstrated by *MacMunn* in acid extracts of skeletal muscles. *Keilin*¹² later attempted the purification from baker's yeast, but the product obtained was very impure¹³. In 1936 *Theorell*¹⁴ designed a method for preparing beef cytochrome c; heart muscle was extracted with sulphuric acid, neutralized and fractionated, at one stage at 60°, with ammonium sulphate followed by adsorption steps on barium sulphate or cellophane; the iron content of this preparation was 0.34 %. The following year *Keilin* and *Hartree*³³ introduced a method which for a long time has been used as a standard method for preparing cytochrome c (cytochrome c prepared according to this method will hereafter be referred to as *K.-H.* preparation). They used trichloroacetic acid instead of sulphuric acid for extraction, and after neutralisation followed by fractionation with ammonium sulphate, the cytochrome was finally precipitated with trichloroacetic acid. *Keilin* and *Hartree* claimed a 0.34 % iron content for their preparation. A further purification was achieved by *Theorell* and *Åkeson*¹⁷ who were able, with the aid of preparative electrophoresis, to raise the iron content to 0.43 %. *Keilin* and *Hartree*³⁴ later modified their original method by adding a fractionation step with ammonium sulphate in alkaline solution and claimed that they thus obtained a preparation with the same iron content as that of *Theorell* and *Åkeson*. The present author, however, has not invariably obtained such pure preparations as claimed by *Keilin* and *Hartree*, for their modified method. *Roche et al.*³⁵ took advantage of the heat stability of cytochrome c and by boiling a *K.-H.* preparation for three minutes in 10 % ammonium sulphate raised the iron content to 0.40 %.

In 1950, *Paléus* and *Neilands*²⁵ introduced a new method for purifying cytochrome c with the aid of ion exchange resins. In this procedure a weak cation exchanger, Amberlite IRC-50 or XE-64, is used and advantage is taken of the high isoelectric point of the cytochrome for its purification. A *K.-H.* preparation is put on a specially prepared column at pH 9.0, and a golden coloured fraction is then seen to separate from the red cytochrome c top layer. Slowly the cytochrome begins to move down the column; a reduced fraction (called "fraction I") preceeds and finally

separates from the oxidized form. The latter always shows the highest iron content, in the case of beef cytochrome 0.42 % to 0.46 %. This oxidized fraction (hereafter referred to as "fraction II") was consequently used for the subsequent studies of the composition and physico-chemical studies of cytochrome c. In 1952 *Paléus*²⁷ described a method for purifying cytochrome c by paper electrophoresis. The iron content in these preparations, however, was never as high as in the ion exchange method of *Paléus* and *Neilands*. Recently, the chromatographic behaviour of cytochrome c on cation exchangers has been extensively investigated by *Margoliash*³⁶. He verified the results of *Paléus* and *Neilands*, and demonstrated that the use of trichloroacetic acid for the extraction and the final precipitation in the *K.-H.* procedure produces a fraction of cytochrome c, which is inactive in both the cytochrome-oxidase and succinic-oxidase systems but is spectroscopically indistinguishable from active cytochrome c. "Fraction II" used in this investigation was, however, on the basis of *Margoliash*' investigation, assumed to be as active in the above mentioned systems as the starting material, calculated per mole cytochrome iron. A modification of the preparation method according to *Paléus* and *Neilands* was recently introduced by *Lofftfield* and *Bonnichsen* and used by *Boeri* and *Tosi*³⁷. They avoided extraction with trichloroacetic acid and obtained the cytochrome, mainly in the ferrous form, with an iron content varying between 0.34–0.38 %. *Boeri* and *Tosi*³⁷ have studied this preparation in connection with the effect of salts on the autoxidation of cytochrome c, and demonstrated that it did not differ very much from a *K.-H.* preparation.

There has been much discussion as to the nature of pure cytochrome c. *Keilin* and *Hartree*^{33, 34} claimed that their preparation with 0.34 % iron was pure cytochrome. They considered, even after *Theorell* and *Akeson*¹⁷ prepared cytochrome c with 0.43 % iron, that intracellular cytochrome c contained 0.34 % iron. This idea is not tenable since *Margoliash*³⁶ has shown that the activity of the purest fraction of cytochrome c obtained on the ion exchanger is 27 % greater than that of a *K.-H.* preparation in the cytochrome oxidase and succinic oxidase systems, calculated per total protein present in the preparation. The determination of iron is obviously very important in the study of cytochrome c. Different investigators have used various methods of iron analyses, but usually the accuracy of the methods has not been reported. It is therefore difficult to compare the iron values in the literature. The method used in this work and the experimental errors involved will be described below.

For a long time, cytochrome c containing 0.43 % iron was considered as pure. Several investigators^{35, 38}, however, suspected from their experimental data that the iron content of pure beef cytochrome might be higher than 0.43 %. Recently, *Tint* and *Reiss*³⁹ confirmed this suspicion. They made a comparative electrophoretic investigation of cytochrome c from beef, horse, pig and chicken prepared according to the prescriptions of *Keilin* and *Hartree*^{33, 34}. Already their starting material of horse cytochrome showed an iron content of 0.45 %. In the other animal cyto-

chromes *Tint* and *Reiss* were able, with the help of the electrophoretic pattern, to calculate the iron content, which in the case of beef was 0.45 %. It is difficult to say whether this value represents pure beef cytochrome c; this question will be further discussed in the chapter on its chemical composition. Another measure of purity is the $\epsilon_{550}/\epsilon_{280}$ ratio²⁵, which is especially valuable for following the steps in the preparation procedure. Paper electrophoresis²⁷ at pH 7.0 revealed only one component as did a *Tiselius* electrophoresis²⁵ of our material at pH 10.5. This fact was later verified by *Neilands*⁴⁰. The ultracentrifuge revealed one single boundary. The sedimentation constants of beef, chicken and salmon cytochrome³⁰ were 1.64, 1.63, 2.09 S, respectively.

Chemical composition

1. The results of other investigators

The first elementary analysis on cytochrome c was reported by *Theorell* in 1936¹⁴; he found 0.34 % iron, 1.18 % sulphur and 14.4 % nitrogen in his beef cytochrome c preparation. The following year, *Keilin* and *Hartree*³³ analyzed beef cytochrome and obtained the same iron content as did *Theorell*. Moreover, they found 1.1 % cystine (< 1 residue/molecule cytochrome c), 7.8 % histidine (> 8 residues/molecule) and 9.1 % lysine (> 10 residues/molecule). The most extensive chemical analysis, however, was made on beef cytochrome by *Theorell* and *Åkeson*¹⁸. As mentioned above it contained 0.43 % iron, which corresponds to a minimal molecular weight of 13,000. This value is in accordance with 13,200 obtained from sedimentation and diffusion experiments⁴¹ and shows that cytochrome c contains only one iron atom per molecule. The sulphur content of *Theorell* and *Åkeson*'s preparation was 1.466 % (= 6 atoms per molecule cytochrome) and the nitrogen content 15.36 % (= 143 atoms per molecule). It may also be mentioned that the preparation contained 3 histidines and around 22 lysines, the latter being responsible for the basic character of cytochrome c. Since the investigation of *Theorell* and *Åkeson*, beef cytochrome c has been analyzed for the sulphur containing amino acids and histidine, the former by *Åkeson*²⁸ and the latter by *Paul*⁴², and their results will be discussed later. *de Barbieri* and *Zamboni*⁴³ have made a complete amino acid analysis of horse cytochrome containing 0.456 % iron, prepared according to *Paléus* and *Neilands*. They found only 0.87 mole of histidine. This, together with discrepancies with the content of other amino acids suggest faulty analytical methods. In contrast, *Margoliash*⁴⁴ for example recently reported 4 moles of histidine in horse cytochrome with an iron content of 0.46 % prepared in the same way.

Besides the elementary and amino acid analysis of cytochrome c, its prosthetic group has been extensively studied. As mentioned in the introduction, *Paul*^{19, 20} recently succeeded in splitting cytochrome c with silver salts into a colourless protein moiety and an optically active isomer of haematohemin. Just recently, *Morrison et al.*⁴⁵ were able,

out of the reduced and oxidized fraction, respectively of beef cytochrome c prepared according to *Margoliash*, to isolate, after splitting the preparation with silver, two closely related haematohemins. This finding is hard to explain and we should wait for the results of their future investigations.

2. Own investigations

Elementary analysis

a. The iron determinations were performed according to a modification of *Lorber's* colorimetric method used in this laboratory and described by *Paul*⁴⁶. The experimental error of the method was calculated (table I) and applied to the iron and minimal molecular weight determination (table II). As is seen from table II an experimentally iron content of 0.43 % obtained in a single determination means that the real iron content may be somewhere between 0.41 and 0.45 %, if a variation of $2 \times s$ is used. This error will also influence the minimal molecular weight determinations to a corresponding degree (table II). The mean values of the iron determinations were 0.43, 0.41 and 0.45 % for beef, chicken and salmon cytochrome, respectively, and the corresponding minimal molecular weights, assuming one atom of iron per molecule, 13,000, 13,600 and 12,400. The iron value of beef cytochrome is in accordance with the results of *Theorell* and *Åkeson* in 1941, but like the iron value of chicken cytochrome, lower than those reported by *Tint* and *Reiss*³⁹. We have, however, verified the claim of these authors that chicken cytochrome contains a lower percent of iron than beef cytochrome²⁶. Salmon cytochrome does not seem to be prepared previously. The above iron values for beef and salmon cytochrome are lower than those presented by *Paléus* and *Neilands*²⁵ in 1950, a fact which might be explained by a difference in the ion exchanger, but it should also be remembered that those earlier values were derived from single iron analyses on single preparations.

Table I. Estimation of errors in determination of dry weight and of spectrophotometry

	Dry weights	Spectrophotometrical readings
Number of duplicate samples.....	16	14
Average of samples ($\bar{x}$).....	3.549	0.1545
Mean difference ($\bar{d} \pm e_{\bar{d}}$).....	0.00625 ± 0.02050	-0.00064 ± 0.00124
Standard deviation of difference (s_d)....	0.0820	0.00465
Standard deviation of single sample (s_x) ¹	0.0580	0.00329
Standard deviation in per cent of mean ($s_x \%$) ²	1.63 %	2.13 %
Standard error of duplicates in per cent of mean ($s_{\bar{x}} \%$) ³	1.15 %	1.51 %

$$^1 s_x = \frac{1}{\sqrt{2}} \cdot s_d$$

$$^2 s_x \% = s_x \cdot \frac{100}{\bar{x}}$$

$$^3 s_{\bar{x}} \% = \frac{1}{\sqrt{2}} \cdot s_x \%$$

Table II. Experimental error of iron determinations and of minimal molecular weight

	Iron	Molecular weight
Standard deviation of single sample (s) based on two spectrophotometric readings and one dry weight determination in per cent of mean ¹	2.22 %	2.22 %
<i>Variation between single samples</i>		
Mean $\pm 2 \cdot s$ (comprising 19 cases out of 20, $P = 0.05$)	0.43 % $\pm 2 \cdot 0.0095$ (0.411 — 0.449)	13.000 $\pm 2 \cdot 290$ (12.400 — 13.600)
Standard error (e) of mean of 7 samples ² in per cent of mean	0.84 %	0.84 %
<i>Variation between means</i>		
Mean $\pm 2 \cdot e$ (comprising 19 out of 20 cases, $P = 0.05$)	Beef 0.43% $\pm 2 \cdot 0.00361$ (0.423 — 0.437) Chicken 0.41% $\pm 2 \cdot 0.00345$ (0.403 — 0.417) Salmon 0.45% $\pm 2 \cdot 0.00378$ (0.442 — 0.458)	13.000 $\pm 2 \cdot 110$ 12.800 — 13.200) 13.600 $\pm 2 \cdot 115$ (13.400 — 13.800) 12.400 $\pm 2 \cdot 105$ 12.200 — 12.600)

¹ $s = \sqrt{s_w^2 + s_B^2}$, where s = experimental error of iron or molecular weight.

s_w = error of dry weight determination (1.63 %, table I), and s_B = error of spectrophotometry (1.51 %, table I).

² $e = \frac{s_x}{\sqrt{7}}$

b. *Nitrogen* determinations were made according to the micro-Kjeldahl procedure on beef (0.45 % Fe), chicken (0.44 % Fe) and salmon cytochrome (0.45 % Fe) and gave the values 15.00, 15.30 and 15.56 % nitrogen respectively. *Theorell* and *Åkeson*¹⁸ in 1941 found 15.37 % nitrogen in their purest preparation, corresponding to 143 nitrogen atoms per iron atom; *Paul*²⁰, in 1951 obtained 15.39 % nitrogen (145 nitrogen atoms per iron atom) in a preparation of beef cytochrome (0.424 % Fe). It is remarkable that all of the preparations of *Paléus* and *Neilands* have a somewhat lower nitrogen content.

c. *Sulphur* content. *Theorell* and coworkers^{14, 18, 28} have reported a value of 6 sulphur atoms per molecule beef cytochrome c. The distribution of these S-atoms was extensively investigated by *Åkeson*²⁸. He could, however, only account with certainty for four sulphur atoms, two belonging to cysteine residues (involved in the thioether linkages) and to two methionine residues. The beef cytochrome of the author's own investigation contained 4 S-atoms as determined by three different methods²⁹. The polarographic investigations did not reveal any free SH- or S-S groups. The four sulphur atoms of bovine cytochrome c are distributed among two methionine residues and two cysteine residues, belonging to the two thioether bridges demonstrated by *Theorell*^{15, 16}.

Amino acid analysis

a. *Paper chromatography* of the amino acids were made on acid hydrolysates according to a method indicated by *Maehly and Paléus*⁴⁷. Tryptophane, however, was assayed on an alkaline hydrolysate. All the usual amino acids except oxyproline were to be found in all the species. Leucin and isoleucin are not separated by the paper chromatographic method used. Weak spots were given by phenylalanine, histidine and methionine. It is known from the investigations of *Ågren and Nilsson*⁴⁸ that methionine and histidine give weak spots with ninhydrin when phenol and collidine are used as solvents. Lysine gave a very strong spot as was to be expected by the analysis of *Theorell and Åkeson*¹⁸. The alkaline hydrolysates showed tryptophane to be present in all the three species, especially in the hydrolysate of salmon cytochrome, which gave a strong tryptophane reaction. The paper chromatographic analysis of the cytochromes shows the same amino acids to be present as in the quantitative analysis of beef cytochrome by *Theorell and Åkeson*¹⁸. It also shows that the amino acid composition in the investigated species is qualitatively very similar.

b. *Histidine* determinations. *Theorell's* and *Åkeson's*¹⁸ and *Paul's*⁴² finding of 3 histidines per mole in beef cytochrome c was confirmed by the present author in his own determinations. The same number of histidine residues were found in chicken cytochrome, but only two in salmon. Recently, *Margoliash*⁴⁴ reported that purified horse cytochrome contains 4 histidine residues, one of which is N-terminal; of the three remaining histidines, two do not react with 1:2:4-fluorodinitrobenzene while the last one reacts with its imidazole group.

c. *Methionine* determination. This amino acid was determined in connection with the sulphur distribution in bovine cytochrome c. Two residues were found, thus confirming the results of *Åkeson*²⁸. Methionine is also present in chicken and salmon cytochrome, but no quantitative analysis was made on these species.

Spectrophotometric investigations

Already *MacMunn*² had indicated the approximate positions of the absorption bands of cytochrome c in the visible region. The exact positions of these bands were later determined by *Keilin*³ who obtained two rather broad bands at 550–570 m μ and 520–540 m μ for oxidized cytochrome c, and two sharp bands at 550 m μ and 521 m μ for the reduced form. He also showed that the cytochrome c spectrum differs very little from one organism to another. Cytochrome c, as mentioned in the introduction, has a haemochromogen structure⁴, and accordingly shows one absorption band around 400 m μ (the Soret band*) and two bands in the 500–600 m μ region (the band observed around 550 m μ is the α -band and that observed around 520 is the β -band). *Keilin*⁴⁹ showed that the absorption spectrum of cytochrome c does not undergo any appreciable change on

* Named after the French scientist *Soret*⁵⁰, who first observed it in haemoglobin.

Table III. Absorption spectra of ferro-cytochromes c

	$\epsilon_{550 \text{ m}\mu}$	$\epsilon_{535 \text{ m}\mu}$	$\epsilon_{520 \text{ m}\mu}$	Soret-band	pH	Reference
Salmon	26,250	7,020	15,030	134,100	6.0	own investigation ²⁶
Chicken	26,910	7,260	14,870	132,100	6.0	»
»	26,980	7,150			6.0	<i>Tint & Reiss</i> ³⁹
Beef	26,880	7,040	15,470	142,200	6.0	own investigation ²⁵
»	28,100		16,900	143,200	7.5	<i>Theorell</i> ¹⁴
»	26,000	7,040	15,540		5.25	<i>Drabkin</i> ⁵⁵
»	26,400	7,030			6.0	<i>Tint & Reiss</i> ³⁹

$$\epsilon = \frac{1}{c \cdot d} \cdot \log I_0/I$$

I_0 and I are the intensities of the incident and transmitted light respectively

c = concentration of cytochrome (g. atoms Fe/l)

d = optical path (cm)

treatment with pyridine and alkali. In accordance with this *Theorell* and *Åkeson*¹⁸ reported the α -band of the pyridine haemochromogen of beef cytochrome c at 550 m μ , at which wavelength ferrocytochrome c itself shows a maximum. The same result has been obtained by the present author on the cytochromes from chicken and salmon, and by *Vernon* and *Kamen*⁵¹ on cytochrome c from *Rhodospirillum rubrum*. All these results indicate an identical prosthetic group in cytochrome c from different sources. The Soret-band region of cytochrome c was first investigated by *Warburg* and *Negelein*⁵² and later by *Dixon et al.*¹³ and others^{14, 53}; the band is situated at 407 m μ in the oxidized and at 415 m μ in the reduced cytochrome. Both ferro- and ferricytochrome also show an absorption maximum at 280 m μ due to the presence of aromatic amino acids. Besides this maximum in the ultraviolet range, ferrocytochrome has an absorption maximum at 316 m μ , attributed by *Paul* and *Theorell*⁵⁴ to its haemochromogen nature. Due to this same cause ferricytochrome shows a maximum at 364 m μ according to the same authors.

The first complete spectrum of bovine cytochrome c was presented by *Theorell*¹⁴. Since that time many authors have contributed spectral data. In table III are listed the molar extinction coefficients per gram atom of iron of cytochrome c from beef, chicken and salmon at different wavelengths. Available values for the same species by other investigators are also listed. As is seen from the table the differences between the various species in the visible range are small, recently emphasized by *Neiland's* investigations⁴⁰ on cytochrome c from rust fungus. The extinctions at 280 m μ vary, however, due to the different content of aromatic amino acids in the species²⁶.

Acid-base titrations

In connection with studies of the haem linkage of cytochrome c, *Theorell* and *Åkeson*¹⁸ made titration curves on bovine cytochrome c and similar

experiments were later carried out by *Paul*⁴². He also titrated the protein moiety of bovine cytochrome after splitting with silver. In the present work, potentiometric titration curves on cytochrome c from different species have been compared²⁶, especially in the pH range where the histidine group is usually titrated, *i.e.* around pH 6.

The evaluation of titration curves of proteins is a rather difficult problem. This is due to the rather great number of ionizable groups within the proteins and to our incomplete understanding of the mutual interactions between these groups, on the one hand, and the interactions between them and the solvent with its ions on the other. Nevertheless, valuable information about the structure of different proteins has been gained by titration curves, especially when these were correlated with available analytical data^{56, 57}. A titration curve of a protein may be looked upon as a complex dissociation curve of ionizing groups of different kinds, formed by the constituent amino acids. The pK's of free groups of amino acids forming part of a polypeptide chain are usually not shifted very far from the pK's of the corresponding groups of the free amino acids. *Cannan*⁵⁸ has analyzed the titration curve of gelatin into the following regions characteristic of the titration of the present ionizable groups: a) pH 1.5–6.0, carboxyl groups; b) pH 6.0–8.5, imidazole groups; c) pH 8.5–11.5, α -amino, phenolic and sulfhydryl groups; d) > pH 11.5, guanidine groups of arginine. The α -amino groups should also contribute to the central region of the curve. This scheme of interpretation cannot generally be applied without due consideration being taken as to the structure of the protein under investigation, but it is sufficient for a rough evaluation of the titration results.

For the correct evaluation of the α -NH₂- and α -COOH-groups titrated the numbers of the following amino acids should be known: monoamino-dicarboxyl acids (aspartic acid, glutamic acid) and their amides (asparagine and glutamine), lysine- and cystine residues. Finally, there may be ionizing groups present in the prosthetic group of conjugated proteins like cytochrome c. In this particular case the titration curve is still more complicated through the presence of the iron, either di- or trivalent.

It is assumed that the protein will not influence the activity of hydrogen ions⁵⁹ and in the present work no consideration has been given to the influence of the ionic strength. For the analysis of the titration curves the author used a recently developed graphical method²⁶. It was thus possible to demonstrate that in beef and chicken cytochrome one acid equivalent was titrated with a pK of 6.8 and two equivalents with a pK of 5.4 (beef) and 5.5 (chicken). These pK-values were not shifted upon reduction of the cytochromes. In salmon cytochrome one acid equivalent with a pK of 6.5 was titrated. The steep appearance of the curve in the region around pH 5.5 did not allow any detailed analysis but at least two groups were titrated there. On analyzing the titration curve obtained by *Theorell* and *Åkeson*¹⁸ the author found the result of one acid equivalent with a pK of 6.8, a value in close agreement with his own. From *Wyman's* study⁶⁰ of the temperature effect on the titration curve of oxyhaemoglobin, it is known that the imidazole group has a charac-

teristic heat of dissociation and is titrated between pH 5.5—8.5. From similar experiments *Theorell* and *Åkeson*¹⁸ deduced that only one of the three imidazole groups in bovine cytochrome c was titrated within its normal pH-range. The pK of 6.8 found in both the present author's analysis of their titration curve and his own, is attributed to this single group. The same applies in the case of chicken cytochrome c. Further support⁶¹ for these findings is afforded by the electrophoretic studies of cytochrome c by *Theorell*¹⁴ and *Tint* and *Reiss*³⁹, which indicate the presence of ionizable groups around pH 6—7, as evidenced by the change in slope of the mobility curve. The pK of the imidazole group in free histidine is 6.0⁶², but when linked in a peptide chain a shift of the pK' to 6.7—6.8 has been observed^{56, 57, 63}. The single group with a pK' of 6.8 being titrated within its normal range thus very probably belongs to a non haem-linked group^{64, 65}, which is in contrast to the finding of *Paul*⁶⁶. The pK's at 5.4 in beef and 5.5 in chicken cytochrome are attributed to the presence of the two propionic acid residues in the prosthetic group^{15, 26}.

From the titration curves obtained by the present author, it would appear that cytochrome c contains only one histidine group, but the analysis²⁶ revealed three histidine residues in beef- and chicken cytochrome and two in salmon cytochrome. This discrepancy was explained by the fact that the remaining imidazole groups are involved in the haem linkage and their pK's consequently shift out of their normal titration range, as was first suggested by *Theorell* and *Åkeson*¹⁸ for beef cytochrome. Further support for the theory of haem-linked imidazole groups was later obtained from the study of the magnetic and spectrophotometric behaviour of ferricytochrome c in acid solution⁶⁷. Recently, *Margoliash*⁴⁴ gave an account of the four histidines in horse cytochrome c and their reactivity with 1:2:4 fluorodinitrobenzene. He stated that the first of these reacts with its α -amino and imidazole groups and is thus N-terminal*, the second reacts only with the imidazole group and that the third and fourth do not react at all. This is in accordance with the assumption of two haem-linked imidazole groups.

In salmon cytochrome c the haem linkage must be different from that in beef and chicken cytochrome c, since only one imidazole group is available for haem linkage. If the equivalent with a pK' of 6.5 is attributed to a non haem-linked histidine, the other group in the linkage must consist of some other of the constituent amino acids.

*Paul*⁴² found two equivalents more in the protein moiety of beef cytochrome c between pH 4—7 than in the intact cytochrome c and attributed at least one of them to an imidazole group. According to him the other equivalent might belong either to another imidazole group or to a carboxyl group.

* Expression introduced by *Sanger*⁶⁸ for an amino acid residue of a protein or peptide having a free amino group. C-terminal denotes a residue having a free carboxyl group.

Summary

We may summarize the present concept of the structure of cytochrome c in the following way. The prosthetic group is protohaemin. The protein moiety consists of about 100 of the usual amino acids, arranged in one or possibly two polypeptide chains^{44, 69}. No cross-linkage caused by cystine can be present. The prosthetic group is embedded very firmly in a crevice of the protein moiety, made possible on the one hand by the two thioether bridges between the protein moiety and the side chains at both the 2 and 4 positions of the porphin nucleus, and on the other by coordination of the iron atom to two haemochrome-forming groups, probably derived from two histidine residues in the protein moiety. This firm structure of cytochrome c explains its inaccessibility to molecular oxygen and at the same time its possibility of acting as an electron carrier. The present investigation has shown that the haem linkage may differ with the species and that quantitative differences may occur even if the qualitative composition of the amino acid content is very similar in different animal cytochromes.

II. Studies on the Peptic Degradation Product

In 1951 *Tsou*²¹ degraded horse cytochrome c with different proteolytic enzymes in order to ascertain whether modifications of the protein part of an enzyme with a well defined prosthetic group would be reflected in the catalytic or other properties of the enzyme. The peptic degradation product in particular was extensively studied by him. It had a calculated molecular weight of about 2,500, showed an absorption spectrum which in many respects resembled that of native cytochrome c, but differed from the parent product by being autoxidizable and inactive in the succinic and cytochrome oxidase systems. According to *Tsou* it did not show any peroxidatic activity in contrast to the findings of the present investigation³². It is interesting to note that *MacMunn*² more than 50 years ago isolated a peptic degradation product from "myohematin", which showed a haemochromogen spectrum.

The author has made a comparative investigation of the peptic degradation products from different species and showed them to be very similar in composition²⁶. *Tuppy* and *Bodo*^{22, 71} determined the amino acid sequence of the tryptic degradation product of horse cytochrome (this product will hereafter be called "TMc", trypsin modified cytochrome c), studied some of its physico-chemical properties and showed that no structural difference existed in preparations from horse, cow and pig. An investigation by *Tuppy* and *Paléus*³¹ has been concerned with the purification and amino acid sequence determination of a peptic degradation product (this product will hereafter be referred to as "PMc") of cytochrome c from different species. Finally, *Paléus*, *Ehrenberg* and *Tuppy*³² have studied some of the physico-chemical properties of the peptic degradation product from beef cytochrome.

Preparation and purification

Cytochrome c was digested with pepsin by *Tsou*²¹, the following experimental conditions prevailing: pH 1.5—1.7, cytochrome c concentration 1 % and 1 % crystalline pepsin concentration amounting to 0.15 haemoglobin units⁷⁰ per gram of cytochrome. The temperature was 24° and the digestion time 16—20 hrs. These conditions have been retained in the work of *Tuppy* and *Paléus*³¹ with the following alterations: the temperature was varied between 25—28° and the digestion time 20—28 hrs. The proteolysis was interrupted by the addition of NaOH and the reaction product was then fractionated with ammonium sulphate and trichloroacetic acid. The preparation was dissolved in dilute ammonia and dialyzed first against dilute phthalate buffer of pH 5, and then against water, when a final precipitate was obtained. This was supposed by *Tsou* to be a pure product, but when chromatographed on a "Hyflo Supercel" column²², using a mixture of butanol, glacial acetic acid and water as developer, a separation of three bands was obtained. The main fraction was dissolved in as little ammonia as possible and dialyzed against phthalate buffer and water as above. The precipitate was dried and used in the present work.

Chemical composition

a) *The iron content of Tsou's preparation was 2.21 % corresponding to a minimal molecular weight of 2,500. The preparation of Paléus*²⁶ showed a somewhat higher iron content, namely 2.45 %, 2.27 % and 2.35 %, in the case of beef, chicken and salmon, respectively. In the preparations of *Tuppy* and *Paléus*³¹ the mean iron content of three different beef preparations was 2.77 %, and the iron content of one chicken and one salmon preparation 2.76 % and 2.77 %, respectively, corresponding to a minimal molecular weight of 2,010. However, a calculation based on the amino acid composition and the molecular weight of the prosthetic group (protohemin with a hydroxyl group attached to the iron being assumed), gives a value of 1,880.

Table IV. A comparison of the amino acids present in different preparations of the peptic degradation product of cytochrome c.

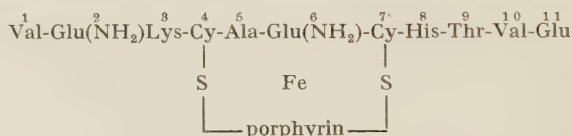
Author	Iron content	Species	Amino acids												
			Glu	Thr	Ala	His	Val	Lys	Asp	Cys	Gly	Leu	Phe	Pro	Ser
<i>Tsou</i> ²¹	2.21 %	horse	+	+	+	+	+	+	+	+	+	+	+	+	(?)
<i>Paléus</i> ²⁶	2.45 %	beef	+	+	+	+	+	+	+	+	+	+	+	+	+
<i>Tuppy</i>	2.77 %	beef ¹	+	+	+	+	+	+	—	+	—	—	—	—	—
and <i>Paléus</i> ³¹			2.99	1.01	1.00	1.11	2.09	1.12							

¹ Amino acid residues per molecule peptide (preparation of *Tuppy* and *Paléus*) are indicated at the bottom of the table.

b) *The amino acid composition* of the PMc obtained by *Tsou*²¹, *Paléus*²⁶ and *Tuppy* and *Paléus*³¹ is listed in table IV. The preparation obtained by the present author differed from that of *Tsou* only by its lower molecular weight and by the presence of serine. It did not reveal any species difference with respect to the qualitative amino acid composition. In the preparations of *Tuppy* and *Paléus*, however, certain amino acids were absent, although they were detectable in *Tsou*'s preparations. They may have been present as impurities in *Tsou*'s preparations but removed from our preparations through the chromatography. As the PMc was prepared by *Tsou* from horse but by *Tuppy* and *Paléus* from beef, any species difference cannot be excluded, but this seems improbable, since *Tuppy* and *Bodo*⁷¹ could not find a species difference between TMc from horse and beef.

The content of some amino acids has been quantitatively determined and the results are listed in table IV. In the present investigation the cysteine has not been quantitatively determined in the peptide, but from the amino acid sequence determinations to be described and from the work of *Tuppy* and *Bodo*²² on TMc, it is known that two residues are present. The three glutamic acid residues are present both in the acid and the amide form, a fact which will be described later.

c) *The amino acid sequence* in the peptides of the present work has been determined by the method of *Sanger*⁶⁸ which has been so successfully applied to the analysis of insulin⁷². In the peptic degradation product of beef and salmon cytochrome, the following sequence was found* (the prosthetic group and the thioether bridges are also indicated):



In the case of chicken the alanine residue at position 5 is replaced by a serine residue. In all the three species one valine residue was found to be N-terminal. In contrast, *Tsou*²¹ found 3.4 α -amino groups per atom of iron in his PMc. His result may be attributed to impurities in his preparation. It is remarkable that the group at position 8 in the near vicinity of the thioether bridge on the C-terminal end of the peptide is a histidine residue, the amino acid which was suggested by *Theorell* and *Åkeson*¹⁸ to be haem-linked in cytochrome c itself. *Tuppy* and *Bodo*²² propose that this residue is one of these haem-linked groups. Of further interest in this connection is residue 3, lysine, which is situated near the thioether bridge on the N-terminal end. The interpositions of alanine and glutamine residues at positions 5 and 6 between the thioether bridges, are in agreement with an α -helix shape of the peptide with 3.7 amino acid residues per turn as shown by *Ehrenberg* and *Theorell*⁷⁴ on steric bond models. The order of the amino acids at the positions 10 and 11 has

* The abbreviations for amino-acid residues are those suggested by *Brand* and *Edsall*⁷³. CyS indicates half a cystine residue.

not been established with certainty but it has been assumed that the order is not different from that in the TMc: $\text{CySO}_3\text{H}-\text{Ala}-\text{Glu}(\text{NH}_2)-\text{CySO}_3\text{H}-\text{His}-\text{Thr}-\text{Val}-\text{Glu}-\text{Lys}$.²²

Physico-chemical properties

Only some of the physico-chemical properties of PMc from beef cytochrome c have been investigated. One of the greatest difficulties in these investigations was the low solubility of the peptide in the pH-range 4.3—7.4, a fact apparently noticed by *Tsou*²¹. This behaviour of the peptide has by *Ehrenberg* and *Theorell*⁷⁴ been attributed to the formation of intermolecular bonds at the proximity of the isoelectric point (about pH 5). Thus the precipitation of the peptide which occurred in this pH range probably caused the peculiar S-shape of the titration curve. About 7 equivalents were titrated between pH 2—11, which is in fair agreement with the given structure. There was no influence of chloride ions on the absorption spectrum at concentrations of 0.001—0.3 M and at pH 2 and 3, which is in contrast to observations made on intact cytochrome⁶⁷. This may be due to the lack of the firm structure around the haem in cytochrome c. Because of the low solubility, the spectrum of the peptide was studied only in the Soret band region between 385 $m\mu$ and 415 $m\mu$, where the light absorption is high. The results indicated the presence of three different forms of the peptide, namely one acid, one intermediate and one alkaline. The acid compound had a fairly sharp absorption maximum, situated at 395 $m\mu$ with $\beta = 41 \cdot 10^{-7} \text{ cm}^2/\text{mole}$. The paramagnetic susceptibility is $11,900 \cdot 10^{-6} \text{ cgs emu}$, indicating the presence of 5 odd electrons in the iron atom, the bonds being essentially ionic. Thus acid PMc very much resembles the acid type II of ferri-cytochrome c, described by *Theorell* and *Åkeson*¹⁸. The alkaline form has its absorption maximum at 406 $m\mu$ with $\beta = 24 \cdot 10^{-7} \text{ cm}^2/\text{mole}$, which is nearly identical with the corresponding values of neutral cytochrome c, 407 $m\mu$ and $\beta = 26 \cdot 10^{-7} \text{ cm}^2/\text{mole}$. Also the paramagnetic susceptibilities, $2,080 \cdot 10^{-6} \text{ cgs emu}$ and $2,120 \cdot 10^{-6} \text{ cgs emu}$, for alkaline PMc and cytochrome c, respectively, are closely coincident, indicating the presence of only one odd electron and covalent bonds in both cases. The interpretation of the intermediate compound is more difficult. It has the Soret band maximum at 396 $m\mu$ with $\beta \sim 30 \cdot 10^{-7} \text{ cm}^2/\text{mole}$. No close analogy could be drawn with any compound of native cytochrome c. Its paramagnetic susceptibility could possibly correspond to three odd electrons and it may be pointed out that the existence of iron compounds with three odd electrons has been established but little is known of the bonds involved⁷⁵.

If the absorbancy of a certain compound is plotted against pH, wavelength and concentration remaining constant, a "spectrophotometric titration curve" is obtained. Two such curves were obtained, at readings of 395 $m\mu$ and 410 $m\mu$, which fitted satisfactorily with normal dissociation curves, with $n=1$ and $\text{pK}=3.4$, and $n=1$ and $\text{pK}=5.8$, respec-

tively. From the study of ferricytochrome c in acid solution⁶⁷ it is known that 2 protons, probably derived from histidine residues, are simultaneously released at a pK of 2.12. In PMc, however, only one histidine is present, and the protons are, as might be expected, not released simultaneously, verified by the above results. The nature of the groups to which the above pK-values refer, has not been unambiguously indicated by the present investigations. *Tuppy* and *Bodo*²² have suggested that the histidine residue in TMc is one of the haem-linked imidazole groups of native cytochrome c. From the titration curve of PMc it cannot be decided if this group has a pK around 3.5 or 6.3. If, however, the pK of the propionic acid residues of the prosthetic group are supposed to be titrated at the same pK as in cytochrome c (~ 5.5)²⁶, the pK of histidine must be shifted down to as far as 3.5 in order to obtain the same degree of coincidence between the theoretical and experimental curves. A haem-linked histidine group in PMc would also be expected to have a pK lower than the normal value (5.5—8.0), but higher than that of ferricytochrome c (pK = 2.12). From the work of *Keilin*⁷⁶, *Rosenberg* and *Clark*⁷⁷, and *Ehrenberg* and *Theorell*⁷⁴, the great haemochrome-forming ability of histidine has been demonstrated. Further support for imidazole as a haem-linked group has been obtained by *Ehrenberg* and *Theorell*⁷⁴ in their use of steric bond models for building chains of PMc, an α -helix fitting most suitably with the known structure of the peptide. It is suggested that the group with a pK of 5.8 is the ϵ -amino group of lysine or the α -amino group of valine, which is in agreement with the assumption of the presence of the imidazole group with a pK of 3.4. The shift of the pK of either a lysine or a valine group from their normal titration range of around 10 and 9, respectively, to 5.8 may be attributed to the haem-linked structure proposed.

Summary

Cytochrome c from beef, chicken and salmon has been digested with pepsin and the degradation product isolated and purified. The amino acid sequence was found to be the same in the beef and salmon peptide, but the alanine residue present in these two peptides was replaced by a serine residue in the chicken peptide. Acid-base titrations, spectrophotometric and magnetic investigations were made and the result correlated with those of cytochrome c.

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References

1. MacMunn, C. A., *J. Physiol.* **5** (1882—84) *Proc.* XXIV.
2. MacMunn, C. A., *Phil. Trans. Roy. Soc. (London)* **177** (1886) 267.
3. Keilin, D., *Proc. Roy. Soc. (London) B* **98** (1925) 312.
4. Anson, M. L. and Mirsky, A. E., *J. Physiol.* **60** (1925) 50, 161.
5. Hoppe-Seyler, F., *Ber.* **3** (1870) 229.
6. Pauling, L. and Coryell, C. D., *Proc. Nat. Acad. Sc.* **22** (1936) 159.
7. Warburg, O., *Heavy Metal Prosthetic Groups and Enzyme Action*, Oxford, At The Clarendon Press, 1949.
8. Keilin, D. and Hartree, E. F., *Proc. Roy. Soc. (London) B* **127** (1939) 167.
9. Ball, E. G., *Biochem. Z.* **295** (1938) 262.
10. Slater, E. C., *Ann. Rev. Biochem.* **22** (1953) 17.
11. Leach, S. J., *Advances in Enzymol.* **15** (1954) 1.
12. Keilin, D., *Proc. Roy. Soc. (London) B* **106** (1930) 418.
13. Dixon, M., Hill, R. and Keilin, D., *Proc. Roy. Soc. (London) B* **109** (1931/32) 29.
14. Theorell, H., *Biochem. Z.* **285** (1936) 207.
15. Theorell, H., *Biochem. Z.* **298** (1938) 242.
16. Theorell, H., *Enzymologia* **6** (1939) 88.
17. Theorell, H. and Åkeson, Å., *Science* **90** (1939) 67.
18. Theorell, H. and Åkeson, Å., *J. Am. Chem. Soc.* **63** (1941) 1804.
19. Paul, K. G., *Acta Chem. Scand.* **4** (1950) 239.
20. Paul, K. G., *Acta Chem. Scand.* **5** (1951) 389.
21. Tsou, C. L., *Biochem. J. (London)* **49** (1951) 362, 367.
22. Tuppy, H. and Bodo, G., *Monatsh.* **85** (1954) 1024.
23. Vickery, H. B., *J. Biol. Chem.* **144** (1942) 712.
24. Porter, R. R. and Sanger, F., *Biochem. J. (London)* **42** (1948) 287.
25. Paléus, S. and Neilands, J. B., *Acta Chem. Scand.* **4** (1950) 1024.
26. Paléus, S., *Acta Chem. Scand.* **8** (1954) 971.
27. Paléus, S., *Acta Chem. Scand.* **6** (1952) 969.
28. Åkeson, Å., *Acta Physiol. Scand.* **4** (1942) 362.
29. Paléus, S., *Acta Chem. Scand.* **9** (1955) 335.
30. Ehrenberg, A. and Paléus, S., *Acta Chem. Scand.* **9** (1955) 538.
31. Tuppy, H. and Paléus, S., *Acta Chem. Scand.* **9** (1955) 353.
32. Paléus, S., Ehrenberg, A. and Tuppy, H., *Acta Chem. Scand.* **9** (1955) 365.
33. Keilin, D. and Hartree, E. F., *Proc. Roy. Soc. (London) B* **122** (1937) 298.
34. Keilin, D. and Hartree, E. F., *Biochem. J. (London)* **39** (1945) 289.
35. Roche, J., Derrien, Y., Cahnmann, J., *Compt. rend. soc. biol.* **140** (1946) 146.
36. Margoliash, E., *Biochem. J.* **56** (1954) 529, 535.
37. Boeri, E. and Tosi, L., *Arch. Biochem.* **52** (1954) 83.
38. Rosenthal, O. and Drabkin, D. L., *J. Biol. Chem.* **149** (1943) 437.
39. Tint, H. and Reiss, W., *J. Biol. Chem.* **182** (1950) 385, 397.
40. Neilands, J. B., *J. Biol. Chem.* **197** (1952) 701.
41. Paul, K. G. in Sumner, J. B. and Myrback, K. (Ed.), *The Enzymes*, Academic Press Inc., New York, 1951, Vol. II, Part I, p. 375.
42. Paul, K. G., *Acta Chem. Scand.* **5** (1951) 379.
43. de Barbieri, A. and Zamboni, A., *Boll. Soc. ital. biol. sper.* **27** (1951) 343.
44. Margoliash, E., *Nature* **175** (1955) 293.
45. Morrison, M., Estabrook, R. W. and Stotz, E., *J. Am. Chem. Soc.* **76** (1954) 6409.
46. Paul, K. G., *Acta Chem. Scand.* **2** (1948) 430.
47. Maehly, A. C. and Paléus, S., *Acta Chem. Scand.* **4** (1950) 508.
48. Ågren, G. and Nilsson, T., *Acta Chem. Scand.* **3** (1949) 525.
49. Keilin, D., *Ergeb. Enzymforsch.* **II** (1933) 239.
50. Soret, J. J., *Compt. rend.* **86** (1878) 708.
51. Vernon, L. P. and Kamen, M. D., *J. Biol. Chem.* **211** (1954) 643.
52. Warburg, O. and Negelein, E., *Biochem. Z.* **233** (1931) 486.
53. Keilin, D. and Slater, E. C., *Brit. Med. Bull.* **9** (1953) 89.
54. Paul, K. G. and Theorell, H., *Acta Chem. Scand.* **8** (1954) 1087.
55. Drabkin, D. L., *J. Biol. Chem.* **140** (1941) 373.

56. Cannan, R. K., Kibrick, A. C. and Palmer, A. H., *Ann. N. Y. Acad. Sci.* **41** (1941) 243.
57. Cannan, R. K., Palmer, A. H. and Kibrick, A. C., *J. Biol. Chem.* **142** (1942) 803.
58. Cannan, R. K., *Chem. Rev.* **30** (1942) 395.
59. Alberty, R. A. in *Neurath, H. and Bailey, K., The Proteins*, Academic Press, New York, 1953, Vol. I, Part A, p. 482.
60. Wyman, J., *J. Biol. Chem.* **127** (1939) 1.
61. Abrahamson, H. A., Moyer, L. S. and Gorin, M. H., *Electrophoresis of Proteins*, Reinhold Publ. Corp., New York, 1942, p. 146.
62. Edsall, J. T. in *Cohn, E. J. and Edsall, J. T., Proteins, Amino acids and Peptides*, Reinhold Publ. Corp., New York, 1943, p. 85.
63. Smith, E. L., Kimmel, J. R. and Brown, D. M., *J. Biol. Chem.* **207** (1954) 533.
64. Coryell, C. D. and Pauling, L., *J. Biol. Chem.* **132** (1940) 769.
65. Wyman, J., *Advances in Protein Chem.* **4** (1948), 407.
66. Paul, K. G., *Arch. Biochem.* **12** (1947) 441.
67. Boeri, E., Ehrenberg, A., Paul, K. G. and Theorell, H., *Biochim. et Biophys. Acta*, **12** (1953) 273.
68. Sanger, F., *Advances in Protein Chem.* **7** (1952) 1.
69. Tuppy, H., personal communication.
70. Anson, M. L., *J. gen. Physiol.* **22** (1938) 79.
71. Tuppy, H. and Bodo, G., *Monatsh.* **85** (1954) 1182.
72. Sanger, F. and Tuppy, H., *Biochem. J. (London)* **49** (1951) 463, 481.
73. Brand, E. and Edsall, J. T., *Ann. Rev. Biochem.* **16** (1947) 224.
74. Ehrenberg, A. and Theorell, H., in press.
75. Selwood, P. W., *Magnetochemistry*, Interscience Publ., New York, 1943, p. 169.
76. Keilin, J., *Nature*, **165** (1950) 151.
77. Rosenberg, L. L. and Clark, W. M., *J. Biol. Chem.* **205** (1953) 617.

